Hydrobiologia (2015) 743:199–209
DOI 10.1007/s10750-014-2037-8

PRIMARY RESEARCH PAPER

Evolution of carbaryl resistance in the water flea *Daphnia*: complex interactions between inbreeding, stress, and selection

Ine Swillen · Joost Vanoverbeke · Luc De Meester

Received: 3 June 2014 / Revised: 20 August 2014 / Accepted: 8 September 2014 / Published online: 14 September 2014

Abstract Human impact often leads to reduced population sizes, and populations exposed to anthropogenic stress may suffer reduced evolutionary potential because of loss of adaptive genetic variation and higher risk of inbreeding depression (ID). Here, we exposed naive and carbaryl-selected inbred and outbred subpopulations of *Daphnia magna* to the pesticide carbaryl shortly after birth, and monitored acute (day 1–4) and post-exposure mortality (day 5—second clutch). Overall, acute mortality was lower than post-exposure mortality, indicating predominantly long-term costs of carbaryl exposure. Surprisingly, we found no indication for ID with respect to mortality upon carbaryl exposure. This may be due to more effective purging of deleterious alleles under standard conditions in the more homozygous inbreds as compared to the more heterozygous outbreds. Alternatively, homozygous pesticide resistance alleles in inbreds may render the inbreds an advantage compared to outbreds, where such resistance alleles would more likely occur heterozygotically. Additionally, we found that the capacity to further reduce mortality in response to carbaryl selection tended to be reduced in inbreds compared to outbreds. Our results thus suggest that inbred lineages may cope equally well as outbreds with pesticide stress.

Keywords Inbreeding · Inbreeding depression · *Daphnia* · Pesticide · Selection

Handling editor: Piet Spaak

I. Swillen (✉) · J. Vanoverbeke · L. De Meester
Laboratory of Aquatic Ecology, Evolution and Conservation, Charles Debériotstraat 32, 3000 Louvain, Belgium
e-mail: ine.swillen@gmail.com

J. Vanoverbeke
e-mail: joost.vanoverbeke@bio.kuleuven.be

L. De Meester
e-mail: luc.demeester@bio.kuleuven.be

Introduction

The impact of human-induced changes such as climate change, habitat loss, habitat fragmentation, and pollution on the earth's biota is overwhelming (Palumbi, 2001; Cardinale et al., 2012; Hooper et al., 2012). Human-induced changes often represent strong selection pressures, and the question arises to which degree natural populations have the capacity to genetically track these changes and whether these evolutionary responses impact population, community, and ecosystem dynamics (Urban et al., 2012). Although there is growing evidence that natural populations may show rapid evolutionary responses (Hairston et al., 2005), the capacity for evolutionary adaptation may be reduced because human impact also often results in drastic reductions in population sizes. Small populations may fail to adequately respond to changing environmental

conditions and stress as they suffer from loss of (adaptive) genetic variation (Bakker et al., 2010; Kellermann et al., 2009; Willi & Hoffmann, 2009) and fixation of potentially deleterious alleles through random genetic drift (Reed, 2005). They will also suffer a higher risk of inbreeding depression (Reed & Frankham, 2003). Inbreeding can lead to reduced fitness of offspring (Charlesworth & Charlesworth, 1987, 1999), and the fitness cost of inbreeding depression may be high for individuals, through reduced reproduction and survival. In addition to the direct effects of these fitness costs on population growth, populations also suffer from reduced genetic diversity (Frankham, 2005).

Inbreeding depression has been reported in numerous plant and animal taxa (see review Keller & Waller, 2002). In some species, inbreeding depression has been shown to lower the potential to deal with stressful conditions (e.g., Armbruster et al., 2000; 2005; Fox & Reed, 2011; Larson et al., 2009) and to lead to an increased liability of population extinction (Saccheri et al., 1998; Bijlsma et al., 2000; Wright et al., 2008). In a number of studies, however, no negative effects of inbreeding were reported after recovery from severe population bottlenecks (e.g., Ellegren et al., 1993; Hoelzel et al., 1993, 2002; Groombridge et al., 2009), and in some cases invasive species have been shown to thrive despite going through strong bottlenecks (e.g., Hawley et al., 2008; Kalinowski et al., 2010; Schmid-Hempel et al., 2007). This suggests that there are mechanisms that decrease the fitness costs of inbreeding. Inbreeding may, for instance, result in purging of the genetic load in populations, as the higher degree of homozygosity linked with inbreeding exposes deleterious recessive or partially recessive alleles to selection (Charlesworth & Charlesworth, 1987; Charlesworth et al., 1990). Effective purging can decrease the detrimental effects of inbreeding and increase mean fitness of inbred populations in the case of partial dominance (e.g., Hedrick, 1994; Byers & Waller, 1999). Yet, purging will not restore population fitness in the case of overdominance, and the effectiveness of purging is also dependent on the environment. Further, changing environmental conditions may cause previously concealed genetic load to be expressed, reducing the effectiveness of purging (Bijlsma et al., 1999). Purging may therefore not always be an effective way to decrease the fitness cost of inbreeding in the long term or in variable environments.

In the present study, we quantify how inbreeding interacts with pesticide tolerance in the freshwater zooplankter *Daphnia magna*. The water flea *D. magna* is an often used model organism in both evolutionary ecology (Lampert, 2011; Miner et al., 2012) and ecotoxicology (e.g., Ward & Robinson, 2005; Tsui & Wang, 2006; Villarroel et al., 2009). *Daphnia* are cyclical parthenogens, alternating periods of parthenogenetic reproduction with phases of sexual reproduction. They have a worldwide distribution (Adamowicz et al., 2009) and play a central role in the aquatic food web, being important grazers of phytoplankton and preferred prey of fish (Miner et al., 2012). Natural populations of *D. magna* are frequently passively exposed to pesticides by run-off (Coors et al., 2009). For several reasons, *Daphnia* populations may also be liable to inbreeding. First, during the parthenogenetic phase, clonal selection may lead to strong clonal erosion effectively decreasing genotypic diversity (Gomez & Carvalho, 2000; De Meester et al., 2006; Ortells et al., 2006; Vanoverbeke et al., 2007). In cases of strong clonal selection in relatively small habitats, only a small set of clonal lineages may survive to the end of the growing season, when sexually produced resting eggs are formed, and effective population size may therefore be small, which could lead to increased inbreeding (Vanoverbeke & De Meester, 2010). Second, if new habitats are colonized by single females that subsequently reproduce parthenogenetically, the first round of sexual reproduction will result in selfing (Ebert et al., 2002).

Severe inbreeding depression in *Daphnia* has been reported with respect to survival under benign conditions (De Meester, 1993; Innes, 1989), reproductive output (Deng & Lynch, 1998), competition with outbred genotypes (Ebert et al., 2002) and resistance to parasitism (Ebert et al., 2007). Here we compare the response of outbred and inbred clonal lineages of the water flea *D. magna* when exposed to pesticide stress, both in the absence and presence of prior exposure to the stressor. Starting from a genotype pool derived from an outbred field population and from inbred families derived from isolates of the same population, we imposed strong selection for resistance to the insecticide carbaryl in an experimental evolution trial. We subsequently compared mortality of a representative subset of genotypes isolated from non-selected (i.e., 'naive') and carbaryl-selected inbred and outbred subpopulations upon exposure to a standardized

concentration of carbaryl. Both naive and carbaryl-selected lines were kept for several generations in the laboratory under standardized conditions before the onset of the mortality experiment, which allowed purging (or purifying selection in the case of outbreds) under benign conditions for all populations. With this study, we had two aims: First, we tested whether both naive and carbaryl-selected inbred lines show higher mortality upon exposure to carbaryl than outbred lines, testing for inbreeding depression upon exposure to this pesticide. Second, we tested whether that carbaryl-selected lines of both inbred and outbred clones are less affected by the pesticide as compared to naive lines, thus testing for evolutionary potential and inbreeding depression with respect to this evolutionary potential.

Materials and methods

Figure 1 schematically outlines our experimental approach. It involves three steps: (1) obtaining outbred and inbred (i.e., selfed families) subpopulations of the water flea *D. magna*; (2) a purging—purifying selection/carbaryl selection phase, involving either (2a) purging or purifying selection of isolates obtained from inbred or outbred dormant eggs under laboratory conditions, involving several generations (>12) of culture under benign conditions—we refer to the resulting sets of lineages as being 'naive' as they never were exposed to carbaryl; or (2b) selecting for carbaryl tolerance through exposure of inbred or outbred subpopulations to carbaryl in aquaria—we refer to the resulting sets of lineages as being 'carbaryl-selected'; and a (3) final experiment quantifying mortality of inbred and outbred clonal lineages in the absence and presence of carbaryl. All lineages are derived from the *D. magna* population of Langerodevijver (50°49′42.20″N—4°38′23.69″E), a pristine, pesticide-free pond in a nature reserve in central Flanders. Lineages were hatched from ephippia from a sample of the dormant egg bank obtained by sampling the superficial (upper 3 cm) layer of the sediment. Hatchlings from the natural dormant egg bank were obtained by exposing the dormant eggs to 20°C under a long-day photoperiod (16L:8D) and providing them with fresh medium (aged tap water), stimuli that in combination are known to induce high hatching rates in *D. magna* from the studied region (De Meester and De Jager, 1993). Hatching rates ranged from 60% (family I1 and I2)—90% (family I3 and outbred population). We used the hatchlings from this dormant egg bank to create our outbred subpopulation (average inbreeding coefficient as measured across 13 microsatellite markers $F_{is} = 0.16$) as well as to isolate single clones that were subsequently used to generate inbred families (inbreeding coefficient $F_{is} = 0.5$).

Generating inbred families

Selfed offspring families were obtained by stimulating the production of sexual eggs in monoclonal populations of three clonal lineages (clones 'I1', 'I2,' and 'I3') hatched from the dormant egg bank of Langerodevijver. We induced sexual reproduction by culturing *Daphnia* in 1 l jars without controlling population densities. Cultures were kept at 20°C in aged tap water (24 h, bubbled by air). Jars were cleaned and half of the medium was refreshed twice a week. Cultures were fed 5×10^5 cells/ml of the green alga *Scenedesmus obliquus* daily. The light regime alternated between 5 days of long-day photoperiod (16L:8D) and 2 days of short-day photoperiod (8L:16D). The combination of crowding and changes in photoperiod is known to induce sexual reproduction in *D. magna* (De Meester & De Jager, 1993). The dormant eggs that were produced in these cultures were removed from the jars twice weekly and stored in eppendorf tubes in the dark at 4°C for several weeks before exposing them to hatching conditions. No hatching of dormant eggs occurred in the cultures as all ephippia were removed twice weekly and events such as a cold period or drought are needed to break diapause of dormant eggs of *D. magna* (De Meester & De Jager, 1993).

Purging/purifying selection under benign conditions

Naive clonal cultures (i.e., cultures that were never exposed to stressors before the onset of the experiment) hatched from dormant eggs were kept for minimally 5–6 parthenogenetic generations (but see Fig. 1 for a timeline) in culture under standardized stock conditions in the laboratory (20°C, 16L:8D photoperiod, aged tap water as medium, fed 1×10^5 cells/ml of the green alga *S. obliquus* twice weekly, no control of densities). More specifically, starting from 100 to 294 individual lineages from each subpopulation (three

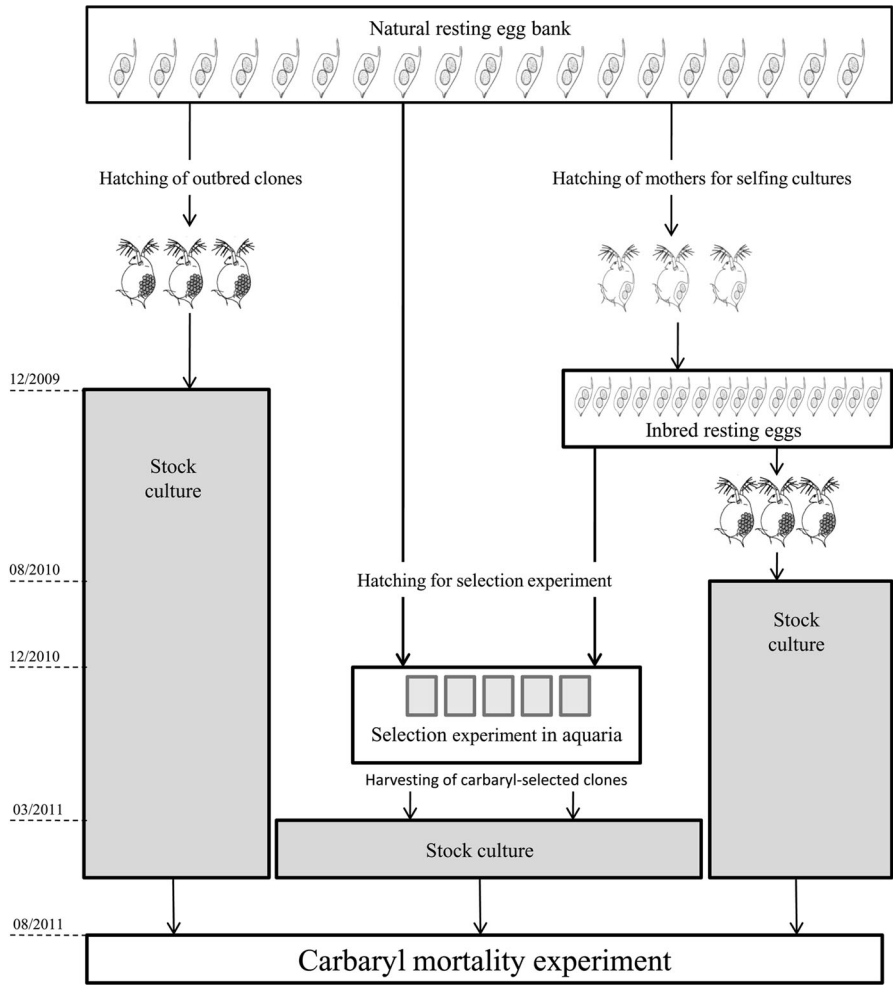

Fig. 1 Schematic overview of the experimental design, which involves three steps: (1) obtaining outbred and inbred (selfed families) subpopulations of the water flea *D. magna* (*top*, indicated by 'hatching'); (2a) purging of 'naive' isolates (obtained from inbred dormant eggs, these lineages were never exposed to carbaryl) in the laboratory, involving several generations of culture under benign conditions (*bars* indicated by 'stock culture') or (2b) exposing 'selected' lineages from each subpopulation to selection by exposure to carbaryl (these lineages were obtained from inbred dormant eggs and immediately exposed to carbaryl in aquaria) (*middle*, indicated by 'carbaryl selection experiment'); and (3) final experiment quantifying mortality of inbred and outbred clonal lineages (*bottom*)

inbred families and one outbred population), we recorded loss of clones due to inviability or sterility during the first eight weeks after hatching (5–6 clonal generations). During these first eight weeks, cultures were cleaned twice per week and fed daily with the green algae *S. obliquus* (1×10^5 cells/ml). By doing so, we were able to record 'purging' of genotypes that suffer from inbreeding depression to such an extent that they are not able to establish monoclonal populations under relatively benign conditions or purifying selection of very weak outbred lineages. Here, we assume loss of lineages was not due to strong selection but that these lineages with very low fitness likely suffer from inbreeding depression caused by homozygosity of strongly deleterious alleles. Purging/purifying selection of clonal lineages during these first eight weeks after hatching was 67% (195/294) for family 'I1', 7% (10/142) for family 'I2'; 57% (102/179) for family 'I3' and 3% (3/100) for outbreds. We thus found strong purging in two out of three of the inbred subpopulations we studied. After 8 weeks, all remaining lineages were kept for several additional months

Table 1 Overview of the number of clonal lines and replicates used in the carbaryl mortality experiment for each group

Group	Naive clonal lines (three replicates per line)	Selected clonal lines (one replicate per line)
Inbred family I1	8	4[a]
Inbred family I2	6	10
Inbred family I3	8	10
Outbreds	8	18

[a] Indicates we reared two replicate isolates per clone for this family, due to low clonal diversity at the end of the aquarium experiment

(Fig. 1 for timeline) in culture under standardized stock conditions in the laboratory (20°C, 16L:8D photoperiod, aged tap water as medium, fed 1×10^5 cells/ml of the green alga *S. obliquus* twice weekly, no control for densities) prior to the experiments.

Selection in the presence of carbaryl

To obtain carbaryl-selected inbred and outbred clones, we subjected clonal lines from 4 different subpopulations (three inbred families and one outbred population; for an overview of the number of clonal lines per subpopulation, see Table 1) to selection by the pesticide carbaryl. Carbaryl is a globally used carbamate insecticide (1-naphtyl methylcarbamate, CAS 63-25-2, purity 99.8%, Sigma-Aldrich, Germany). Carbamate insecticides act as acetylcholinesterase inhibitors and block the transmission of pulses between synapses in the nervous system by preventing the breakdown of acetylcholine, a chemical messenger that is released from the cholinergic nerves and responsible for transmitting the nervous signal through the synaptic cleft. Because of this, a build-up of acetylcholine arises in the synaptic cleft and overstimulates the postsynaptic receptors, thereby preventing the muscles from contracting or relaxing in response to synaptic stimuli (Walker et al., 2006). We started 5 separate 8L aquaria, one for each inbred family and two for the outbred subpopulation. Based on hatching estimates obtained from exploratory hatching experiments, at the start of the selection experiment each aquarium was inoculated by the number of decapsulated dormant eggs that was expected to result in the hatching of approximately 100 clones. This indeed resulted in similar numbers of hatchlings in all aquaria (Swillen, *pers. obs*). The hatched population in each aquarium was exposed to three separate pulses of carbaryl, given at two-week intervals (e.g., Jansen et al., 2011). The first pulse was given 16 days after the start of the experiment, when all first generation individuals (hatchlings) in all aquaria had reached maturity or released their first clutch. The concentration of each pulse was 8 µg/l carbaryl. It has been shown that this concentration induces mortality in juveniles (Jansen et al., 2011). We prepared stock concentrations of carbaryl (1-Naphtyl methylcarbamate, CAS 63-25-2, purity 99.8%, Sigma-Aldrich, Germany) and stored them at −20°C in brown vessels. We fed all aquaria daily with 1×10^5 cells/ml of *S. obliquus* and they were cleaned, and medium was refreshed every 2 days and 24 h after each carbaryl pulse. The selection experiment lasted for 56 days, which is more than four generations of parthenogenetic reproduction (i.e., clonal selection; *D. magna* has an average generation time of about 10–14 days). At the end of the experiment, we sampled 40 individuals per aquarium, and cultured these as monoclonal populations under standardized stock culture conditions. For use in further experiments, we genotyped isolated lineages for 13 microsatellite markers to make sure that they belong to different clones; only individuals with different genotypes as scored by microsatellites were used in the subsequent mortality experiments. This way, we identified 5–17 clones in the inbred subpopulations and 15–20 clones in the outbred subpopulations at the end of the carbaryl selection experiment. The set of microsatellite markers used was found to be sufficient to differentiate multilocus genotypes among all individuals hatched from the dormant egg bank of Langerodevijver in previous analyses (Orsini et al., 2012).

Mortality experiment

From all 8 subpopulations (three inbred families and one outbred population times two conditions: naive and carbaryl-selected settings; outbred clones isolated from the two replicate aquaria in the carbaryl selection experiment were pooled), we exposed 4–18 clonal isolates (see Table 1) to a standardized concentration of carbaryl and monitored mortality. To minimize interference from maternal effects, we cultured the *Daphnia* individually for two generations in 210 ml jars in aged (24 h, bubbled by air) tap water in a temperature-controlled room at 20°C (photoperiod 16L:8D) before the actual start of the experiment.

Medium was refreshed every 2 days, and individuals were fed 1×10^5 cells/ml of *S. obliquus* daily. Per clone, 8–15 s-clutch neonates of the third generation were collected within 24 h after birth and collectively exposed in a 210 ml jar to a first carbaryl pulse with a concentration of 8 µg/l, the same concentration as used in the selection experiment. We refreshed the medium entirely on the second day, and the populations were exposed to a second pulse of 8 µg/l carbaryl on the third day. We assessed acute mortality on the fourth day. On the fifth day, we isolated individuals for each clone and reared them separately in aerated tap water in the absence of carbaryl. We monitored these individuals until death or the release of their second clutch. Jars were cleaned and medium refreshed every 2 days and animals were fed 1×10^5 cells/ml of *S. obliquus* daily. For clones from the naive subpopulations, we reared three individuals separately, while for clones from the carbaryl-selected subpopulations, we reared only one individual, except for the first inbred family (I1), as we only had 4 clonal lineages for this particular family and wanted to increase the power by obtaining a better average estimate for each clone as compared to a better average estimate over clones in the other subpopulations.

Statistics

We calculated two measures of mortality: (1) a measure of acute mortality upon exposure to carbaryl, estimated from cohorts of juveniles during the first 4 days of the experiment, and (2) a measure of post-exposure mortality estimated from a subset of survivors individually cultured until release of the second clutch. For acute mortality, the number of individuals per clone we tested ranged from 8 to 15, while for post-exposure mortality the number of tested individuals per clone ranged from 1 to 3. For both variables, every tested individual either survived or died (binomial response). We analyzed both acute and post-exposure mortality using a two-factorial generalized linear model with the number of dead versus the number of tested individuals per clone (specified as count data with binomial error distribution and logit link function) as the dependent variable, with subpopulation (three inbred and one outbred) and carbaryl selection (naive versus carbaryl selected) as independent categorical factors. In addition to the main analyses, we specified a number of contrasts of interest, which tested the significance of differences between each inbred subpopulation and the outbreds within each selection level (naive or carbaryl selected) on the one hand, and the significance of differences between selection levels within each subpopulation on the other hand. Analyses were done in SAS 9.3 using proc genmod (SAS institute inc., 2002–2010).

Results

Average acute mortality of the different subpopulations ranged from 0 to 25% overall (Fig. 2). There was a significant main effect of subpopulation on acute mortality ($P = 0.0001$, Table 2; Fig. 2) but not of carbaryl selection, nor of the interaction between subpopulation and carbaryl selection. When looking at specific contrasts, before carbaryl selection inbred family 'I2' showed higher mortality than the outbreds (Contrast analysis, $df = 1$, $\chi^2 = 13.75$, $P < 0.001$), whereas the two other inbred families did not (Fig. 2). After carbaryl selection, both inbred families 'I1' and 'I2' differed significantly from the outbreds ('I1', lower mortality than outbreds, $df = 1, \chi^2 = 4.29, P = 0.038$; 'I2', higher mortality than outbreds, $df = 1$, $\chi^2 = 18.27, P < 0.001$, Fig. 2). The response to selection was significant in inbred family I1 (contrast analysis, $df = 1$, $\chi^2 = 4.53$, $P = 0.033$, Fig. 1), with reduced mortality in the selected lines, i.e., after carbaryl selection, but not in the other two inbred families nor in the outbred family.

Average post-exposure mortality ranged from 4.2 to 44.4% (Fig. 2). There was no overall effect of subpopulation or carbaryl selection, but there was a marginally non-significant subpopulation × selection interaction ($P = 0.063$, Table 2; Fig. 2), indicating that the response to selection tended to differ between families. Remarkably, two naive inbred families showed lower post-exposure mortality than the naive outbred population (naive 'I1' vs outbreds, contrast analysis, $df = 1$, $\chi^2 = 5.49$, $P = 0.019$; naive 'I2' vs outbreds, contrast analysis, $df = 1$, $\chi^2 = 5.57$, $P = 0.018$; Fig. 2). However, the difference in mortality between inbreds and outbreds disappeared after carbaryl selection (contrast analyses, all $P > 0.13$), as the carbaryl-selected outbred population showed a tendency to respond to selection by a decrease in post-exposure mortality (marginally non-significant, outbreds before and after selection, contrast analysis,

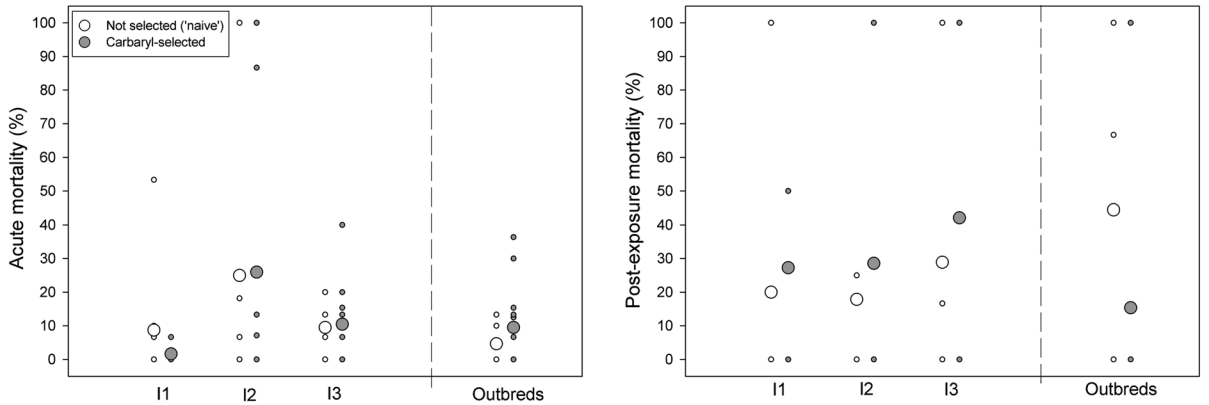

Fig. 2 Acute mortality (day 1–4, %, *left*) and post-exposure mortality (day 5—release of second clutch, %, *right*) of naive (*open symbols*) and carbaryl-selected (*closed symbols*) subpopulations from all families (inbred families I1, I2 & I3, *left*-outbred, *right*). *Large symbols* indicate subpopulation mean mortality values, *small symbols* indicate the mean mortality value of each individual clone. Note *small symbols* may overlap due to identical mean mortality values among clonal lineages

Table 2 Results of generalized linear models testing for the effect of subpopulation (three inbred families, one outbred population), selection (naive or carbaryl selected) and their interaction on acute and post-exposure mortality of clones of *D. magna*

	Acute mortality			Post-exposure mortality		
	df	χ^2	*P*	df	χ^2	*P*
Subpopulation	3	44.93	**<.0001**	3	4.04	0.256
Selection	1	0.34	0.560	1	0.19	0.667
Subpopulation × Selection	3	6.25	0.100	3	7.29	0.063

Significant *P* values are in bold

df = 1, $\chi^2 = 3.09$, $P = 0.078$). One of the three inbred populations also showed a significant response to selection ('I2' before and after selection, contrast analysis, df = 1, $\chi^2 = 4.17$, $P = 0.04$; Fig. 2), but this response was maladaptive, leading to an increase in mortality.

Discussion

Surprisingly, before selection but after purging, naive inbred families are not necessarily more sensitive to carbaryl exposure than outbred populations. Two of our naive inbred families show even lower post-exposure mortality than the naive outbred subpopulation.

Acute and post-exposure mortality

The mortality we observe upon exposure to the used concentration of carbaryl is in line with earlier reports (Jansen et al., 2011). In a study using ten populations of *D. magna*, the EC_{50} for acute mortality ranged from 6.0 to 12.5 µg/l but with strong differences among populations for carbaryl tolerance (Coors et al., 2009). Here, we not only monitored acute mortality but also mortality over a two-week period after exposure to carbaryl. Overall, acute mortality (day 1–4; mortality ranging from 0 to 25%) was lower than post-exposure mortality (day 5 until release of second clutch, i.e., approx. day 15; mortality ranging from 4 to 44%). This is striking because the animals were exposed to carbaryl pulses on day 1 and 3, and were transferred to fresh medium without carbaryl from day 4 onwards. Our observation that post-carbaryl exposure mortality is equally high or higher than acute mortality in *Daphnia* suggests that either early damage interferes with further development or that investment in immediate detoxification (e.g., by activation of short-term cellular responses (Ferrari et al., 2007)) ultimately induces a high cost itself that may not only

have immediate consequences, but could also be important in the longer term through trade-offs in resource allocation (Congdon et al., 2001).

Inbreeding and carbaryl resistance

Strikingly, we found that for two out of three inbred families, post-exposure mortality of naive inbred clones was lower than that of naive outbred clones. We thus found no indication for inbreeding depression with respect to mortality upon carbaryl exposure. The majority of studies suggests that inbreeding depression is stronger under stressful conditions (reviewed in Fox and Reed, 2011), although some studies do not observe an increased effect of inbreeding depression upon exposure to stress (Willi et al., 2007; Fox et al., 2011). Our observation of an absence of inbreeding depression upon exposure to stress may be due to the fact that we reared all clonal lines (both inbred and outbred lines) for several generations in the laboratory before engaging in the carbaryl exposure experiment, which resulted in effective purging (through extinction of clonal lineages) of genotypes with strongly reduced fitness because of expression of homozygous deleterious alleles (Hedrick, 1994). Inbreeding depression in *Daphnia* is indeed strong (Innes, 1989; Deng & Lynch, 1998; Ebert et al., 2002), and loss of inbred lines was generally higher than loss of outbred lines. Survival of clonal lineages during the first 8 weeks after hatching was 33% for family 'I1', 93% for family 'I2'; 43% for family 'I3', and 97% for outbreds. We thus observed strong purging in two out of three of the inbred families we studied. Yet, our observations suggest that once these genotypes are removed from the subpopulations, the remaining inbred genotypes (i.e., the purged inbred subpopulations) surprisingly do not show an increased mortality compared to outbred genotypes under the stressful conditions we provided. We can think of two plausible explanations for our results. First, it could be that due to increased homozygosity purging of sub-lethal alleles under benign conditions was more effective in inbreds than in outbreds. To the extent that such sub-lethal alleles (i.e., sub-lethal under benign conditions) may result in higher mortality upon exposure to stress, this may result in a lower average capacity of outbred compared to inbred clones to deal with carbaryl exposure. If in outbreds the fitness costs of specific deleterious alleles were not sufficiently high to result in mortality under benign conditions but was unmasked after carbaryl exposure (i.e., 'conditional lethals') when higher vigor is needed to cope with stress, this would indeed cause higher average mortality of outbred clones compared to inbred clones, as the latter are more effectively purged from their genetic load. Second, the increased homozygosity of inbred lineages may pre-adapt some of them to cope with carbaryl stress. This would be the case if alleles providing increased resistance to carbaryl in the population are most effective as homozygotes. If these alleles are not extremely rare, inbreeding may result in a significant proportion of inbreds being homozygous for resistance alleles, resulting in a lower mortality upon exposure to carbaryl than observed for outbred genotypes. Similarly, increased resistance to pesticides in inbred lines has been found in the flour beetle, *Tribolium castaneum* (Bengston et al., 1999), and in rice cultivars (*Oryza sativa*) (Bond & Walker, 2011).

Inbreeding and response to carbaryl selection

Our results suggest that an outbred subpopulation of *D. magna* may exhibit a slightly higher capacity to genetically adapt to carbaryl stress, leading to reduced susceptibility to carbaryl exposure after selection (marginally non-significant). Inbred families derived from the same population showed no such tendency of a response or a maladaptive one. Jansen et al. (2011) showed that natural *D. magna* populations harbor evolutionary potential to genetically adapt and show lower acute mortality upon standardized carbaryl pulses. Only inbred family 'I1' showed a significant reduction in acute mortality after selection. For post-exposure mortality, inbred families did not show a response to selection or responded in a maladaptive way, leading to higher mortality. A decreased potential to respond to selection in inbred individuals is in line with findings from other studies (Frankham et al., 1999; Day et al., 2003; Reed et al., 2003). Yet, clones from inbred families in general seem to perform equally well as outbred clones after carbaryl selection. The mechanisms to achieve resistance to carbaryl may therefore be different between inbred and outbred populations and could be subject for future studies: in inbreds, resistance to carbaryl seems to be relatively high in naive animals, perhaps mediated by purging under benign conditions. In contrast, in outbreds, evolution of reduced mortality may be the result of selection upon exposure to carbaryl stress.

General conclusion and implications

A wide variety of studies on the interaction between inbreeding and stress have shown that inbreeding depression is strongly lineage-, species-, and stressor-specific (Reed et al., 2003; Armbruster and Reed, 2005; Kristensen & Sorensen, 2005). Yet, most studies suggest that inbreeding depression is increased under stressful conditions (reviewed in Fox & Reed, 2011). Our results show that, depending on the family, inbred families of the water flea *D. magna* may survive pesticide stress equally well or even better than outbred populations.

Our results suggest that if a population of *D. magna* is colonized by a single genotype, the resulting selfed population following sexual reproduction at the end of the first growing season will not necessarily be outperformed by outbred immigrants, even when the population is exposed to stressful conditions such as pesticides. The resulting population will suffer inbreeding depression, which will likely result in the loss of a substantial number of genotypes upon hatching, but given the vast amount of dormant eggs formed each growing season, the remaining population still has a high numerical advantage over immigrants. Our results show that in the presence of carbaryl the average fitness of this purged population can be equally high as that of outbreds. This is a surprising result indicating that selfed populations may perform reasonably well. However, Haag et al. (2003) showed that inbred populations performed less well in the presence of parasites, which implies that our results may not necessarily hold for all stressors. It nonetheless remains remarkable that exposure to relatively high concentrations of a pesticide does not reveal increased inbreeding depression. In contrast, some inbred families actually outperformed the outbreds in terms of mortality in the presence of the pesticide. We did observe a reduced capacity to further reduce mortality following selection, but even then, the resulting mortality among outbreds upon exposure to carbaryl equaled that of the average inbreds.

Acknowledgments This study was financially supported by FWO project G.229.09 and KU Leuven Research Fund Excellence Financing PF/2010/07.

References

Adamowicz, S. J., A. Petrusek, J. K. Colbourne, P. D. N. Hebert & J. D. S. Witt, 2009. The scale of divergence: a phylogenetic appraisal of intercontinental allopatric speciation in a passively dispersed freshwater zooplankton genus. Molecular Phylogenetics and Evolution 50(3): 423–436.

Armbruster, P. & D. H. Reed, 2005. Inbreeding depression in benign and stressful environments. Heredity 95(3): 235–242.

Bakker, J., M. E. C. van Rijswijk, F. J. Weissing & R. Bijlsma, 2010. Consequences of fragmentation for the ability to adapt to novel environments in experimental *Drosophila* metapopulations. Conservation Genetics 11(2): 435–448.

Bengston, M., P. J. Collins, G. J. Daglish, V. L. Hallman, R. Kopittke & H. Pavic, 1999. Inheritance of phosphine resistance in Tribolium castaneum (Caleoptera: Tenebrionidae). Journal of Economic Entomology 92(1): 17–20.

Bijlsma, R., J. Bundgaard & A. C. Boerema, 2000. Does inbreeding affect the extinction risk of small populations? Predictions from *Drosophila*. Journal of Evolutionary Biology 13(3): 502–514.

Bijlsma, R., J. Bundgaard & W. F. Van Putten, 1999. Environmental dependence of inbreeding depression and purging in *Drosophila melanogaster*. Journal of Evolutionary Biology 12(6): 1125–1137.

Bond, J. A. & T. W. Walker, 2011. Differential tolerance of clearfield rice cultivars to imazamox. Weed Technology 25(2): 192–197.

Byers, D. L. & D. M. Waller, 1999. Do plant populations purge their genetic load? Effects of population size and mating history on inbreeding depression. Annual Review of Ecology and Systematics 30: 479–513.

Cardinale, B. J., J. E. Duffy, A. Gonzalez, D. U. Hooper, C. Perrings, P. Venail, A. Narwani, G. M. Mace, D. Tilman, D. A. Wardle, A. P. Kinzig, G. C. Daily, M. Loreau, J. B. Grace, A. Larigauderie, D. S. Srivastava & S. Naeem, 2012. Biodiversity loss and its impact on humanity. Nature 486(7401): 59–67.

Charlesworth, D. & B. Charlesworth, 1987. Inbreeding depression and its evolutionary consequences. Annual Review of Ecology and Systematics 18: 237–268.

Charlesworth, B. & D. Charlesworth, 1999. The genetic basis of inbreeding depression. Genetics Research 74(3): 329–340.

Charlesworth, D., M. T. Morgan & B. Charlesworth, 1990. Inbreeding depression, genetic load and the evolution of outcrossing rates in a multilocus system with no linkage. Evolution 44(6): 1469–1489.

Congdon, J. D., A. E. Dunham, W. A. Hopkins, C. L. Rowe & T. G. Hinton, 2001. Resource allocation-based life histories: a conceptual basis for studies of ecological toxicology. Environmental Toxicology and Chemistry 20(8): 1698–1703.

Coors, A., J. Vanoverbeke, T. De Bie & L. De Meester, 2009. Land use, genetic diversity and toxicant tolerance in natural populations of *Daphnia magna*. Aquatic Toxicology 95: 71–79.

Day, S. B., E. H. Bryant & L. M. Meffert, 2003. The influence of variable rates of inbreeding on fitness, environmental

responsiveness, and evolutionary potential. Evolution 57(6): 1314–1324.
De Meester, L., 1993. Genotype, fish-mediated chemicals and phototaxis in *Daphnia*. Ecology 74: 1467–1474.
De Meester, L. & H. De Jager, 1993. Hatching of *Daphnia* sexual eggs. 1. Intraspecific differences in the hatching responses of *Daphnia magna* eggs. Freshwater Biology 30(2): 219–226.
De Meester, L., J. Vanoverbeke, K. De Gelas, R. Ortells & P. Spaak, 2006. Genetic structure of cyclic parthenogenetic zooplankton populations – a conceptual framework. Arch Hydrobiol 167(1–4): 217–244.
Deng, H. W. & M. Lynch, 1998. Inbreeding depression and inferred deleterious-mutation parameters in *Daphnia* (vol 147, p. 147, 1997). Genetics 148(1): 537.
Ebert, D., F. Altermatt & S. Lass, 2007. A short term benefit for outcrossing in a *Daphnia* metapopulation in relation to parasitism. Journal of the Royal Society Interface 4(16): 777–785.
Ebert, D., C. Haag, M. Kirkpatrick, M. Riek, J. W. Hottinger & I. Pajunen, 2002. A selective advantage to immigrant genes in a *Daphnia* metapopulation. Science 295: 485–488.
Ellegren, H., G. Hartman, M. Johansson & L. Andersson, 1993. Major histocompatibility complex monomorphism and low levels of DNA fingerprinting variability in a reintroduced and rapidly expanding population of beavers. Proceedings of the National Academy of Sciences of the United States of America 90(17): 8150–8153.
Ferrari, A., A. Venturino & A. M. P. de D'Angelo, 2007. Effects of carbaryl and azinphos methyl on juvenile rainbow trout (*Oncorhynchus mykiss*) detoxifying enzymes. Pesticide Biochemistry and Physiology 88(2): 134–142.
Fox, C. W. & D. H. Reed, 2011. Inbreeding depression increases with environmental stress: an experimental study and meta-analysis. Evolution 65(1): 246–258.
Fox, C. W., R. C. Stillwell, W. G. Wallin, C. L. Curtis & D. H. Reed, 2011. Inbreeding-environment interactions for fitness: complex relationships between inbreeding depression and temperature stress in a seed-feeding beetle. Evolutionary Ecology 25(1): 25–43.
Frankham, R., 2005. Genetics and extinction. Biological Conservation 126(2): 131–140.
Frankham, R., K. Lees, M. E. Montgomery, P. R. England, E. H. Lowe & D. A. Briscoe, 1999. Do population size bottlenecks reduce evolutionary potential? Animal Conservation 2(4): 255–260.
Gomez, A. & G. R. Carvalho, 2000. Sex, parthenogenesis and genetic structure of rotifers: microsatellite analysis of contemporary and resting egg bank populations. Molecular Ecology 9(2): 203–214.
Groombridge, J. J., D. A. Dawson, T. Burke, R. Prys-Jones, M. D. Brooke & N. Shah, 2009. Evaluating the demographic history of the Seychelles kestrel (*Falco araea*): Genetic evidence for recovery from a population bottleneck following minimal conservation management. Biological Conservation 142(10): 2250–2257.
Haag, C. R., O. Sakwinska & D. Ebert, 2003. Test of synergistic interaction between infection and inbreeding in *Daphnia* magna. Evolution 57(4): 777–783.
Hairston, N. G., S. P. Ellner, M. A. Geber, T. Yoshida & J. A. Fox, 2005. Rapid evolution and the convergence of ecological and evolutionary time. Ecology Letters 8(10): 1114–1127.
Hawley, D. M., J. Briggs, A. A. Dhondt & I. J. Lovette, 2008. Reconciling molecular signatures across markers: mitochondrial DNA confirms founder effect in invasive North American house finches (*Carpodacus mexicanus*). Conservation Genetics 9(3): 637–643.
Hedrick, P. W., 1994. Purging inbreeding depression and the probability of extinction – Full-sib mating. Heredity 73: 363–372.
Hoelzel, A. R., J. Halley, S. J. Obrien, C. Campagna, T. Arnbom, B. Leboeuf, K. Ralls & G. A. Dover, 1993. Elephant seal genetic variation and the use of simulation models to investigate historical population bottlenecks. Journal of Heredity 84(6): 443–449.
Hoelzel, A. R., A. Natoli, M. E. Dahlheim, C. Olavarria, R. W. Baird & N. A. Black, 2002. Low worldwide genetic diversity in the killer whale (*Orcinus orca*): implications for demographic history. Proceedings Royal Society of London Series B 269(1499): 1467–1473.
Hooper, D. U., E. C. Adair, B. J. Cardinale, J. E. K. Byrnes, B. A. Hungate, K. L. Matulich, A. Gonzalez, J. E. Duffy, L. Gamfeldt & M. I. O'Connor, 2012. A global synthesis reveals biodiversity loss as a major driver of ecosystem change. Nature 486(7401): 105–108.
Innes, D. J., 1989. Genetics of *Daphnia* obtusa. Genetic load and linkage analysis in a cyclical parthenogen. Journal of Heredity 80(1): 6–10.
Jansen, M., R. Stoks, A. Coors, W. van Doorslaer & L. de Meester, 2011. Collateral damage: rapid exposure-induced evolution of pesticide resistance leads to increased susceptibility to parasites. Evolution 65(9): 2681–2691.
Kalinowski, S. T., C. C. Muhlfeld, C. S. Guy & B. Cox, 2010. Founding population size of an aquatic invasive species. Conservation Genetics 11(5): 2049–2053.
Keller, L. F. & D. M. Waller, 2002. Inbreeding effects in wild populations. Trends in Ecology & Evolution 17(5): 230–241.
Kellermann, V., B. van Heerwaarden, C. M. Sgro & A. A. Hoffmann, 2009. Fundamental evolutionary limits in ecological traits drive *Drosophila* species distributions. Science 325(5945): 1244–1246.
Kristensen, T. N. & A. C. Sorensen, 2005. Inbreeding – lessons from animal breeding, evolutionary biology and conservation genetics. Journal of Animal Science 80: 121–133.
Lampert, W. (ed.), 2011. *Daphnia*: Development of a Model Organism in Ecology and Evolution. International Ecology Institute, Oldendorf/Luhe.
Larson, S., D. Monson, B. Ballachey, R. Jameson & S. K. Wasser, 2009. Stress-related hormones and genetic diversity in sea otters (*Enhydra lutris*). Marine Mammal Science 25(2): 351–372.
Miner, B. E., L. De Meester, M. E. Pfrender, W. Lampert & N. G. Hairston, 2012. Linking genes to communities and ecosystems: *Daphnia* as an ecogenomic model. Proceedings of the Royal Society B 279(1735): 1873–1882.
Orsini, L., K. I. Spanier & L. De Meester, 2012. Genomic signature of natural and anthropogenic stress in wild populations of the waterflea *Daphnia* magna: validation in space, time and experimental evolution. Molecular Ecology 21(9): 2160–2175.

Ortells, R., A. Gomez & M. Serra, 2006. Effects of duration of the planktonic phase on rotifer genetic diversity. Archiv fur Hydrobiologie 167(1–4): 203–216.

Palumbi, S. R., 2001. Evolution – humans as the world's greatest evolutionary force. Science 293(5536): 1786–1790.

Reed, D. H., 2005. Relationship between population size and fitness. Conservation Biology 19(2): 563–568.

Reed, D. H. & R. Frankham, 2003. Correlation between fitness and genetic diversity. Conservation Biology 17(1): 230–237.

Reed, D. H., E. H. Lowe, D. A. Briscoe & R. Frankham, 2003. Fitness and adaptation in a novel environment: Effect of inbreeding, prior environment, and lineage. Evolution 57(8): 1822–1828.

Saccheri, I., M. Kuussaari, M. Kankare, P. Vikman, W. Fortelius & I. Hanski, 1998. Inbreeding and extinction in a butterfly metapopulation. Nature 392(6675): 491–494.

SAS Institute Inc., 2002–2010. SAS 9.3. SAS institute inc., Cary, NC.

Schmid-Hempel, P., R. Schmid-Hempel, P. C. Brunner, O. D. Seeman & G. R. Allen, 2007. Invasion success of the bumblebee, Bombus terrestris, despite a drastic genetic bottleneck. Heredity 99(4): 414–422.

Tsui, M. T. K. & W.-X. Wang, 2006. Acute toxicity of mercury to *Daphnia* magna under different conditions. Environmental Science & Technology 40(12): 4025–4030.

Urban, M. C., J. J. Tewksbury & K. S. Sheldon, 2012. On a collision course: competition and dispersal differences create no-analogue communities and cause extinctions during climate change. Proceedings of the Royal Society B 279(1735): 2072–2080.

Vanoverbeke, J., K. De Gelas & L. De Meester, 2007. Habitat size and the genetic structure of a cyclical parthenogen, *Daphnia* magna. Heredity 98(6): 419–426.

Vanoverbeke, J. & L. De Meester, 2010. Clonal erosion and genetic drift in cyclical parthenogens – the interplay between neutral and selective processes. Journal of Evolutionary Biology 23(5): 997–1012.

Villarroel, M. J., E. Sancho, E. Andreu-Moliner & M. D. Ferrando, 2009. Biochemical stress response in tetradifon exposed *Daphnia* magna and its relationship to individual growth and reproduction. Science of the Total Environment 407(21): 5537–5542.

Walker, C. H., S. P. Hopkin, R. M. Sibly & D. B. Peakall, 2006. Principles of Ecotoxicology, 3rd ed. CRC Press, Taylor and Francis group, New York.

Ward, T. J. & W. E. Robinson, 2005. Evolution of cadmium resistance in *Daphnia* magna. Environmental Toxicology and Chemistry 24(9): 2341–2349.

Willi, Y., S. Dietrich, M. van Kleunen & M. Fischer, 2007. Inter-specific competitive stress does not affect the magnitude of inbreeding depression. Evolutionary Ecology Research 9(6): 959–974.

Willi, Y. & A. A. Hoffmann, 2009. Demographic factors and genetic variation influence population persistence under environmental change. Journal of Evolutionary Biology 22(1): 124–133.

Wright, L. I., T. Tregenza & D. J. Hosken, 2008. Inbreeding, inbreeding depression and extinction. Conservation Genetics 9(4): 833–843.